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## **Olmesartan prevents microalbuminuria in db/db diabetic mice through inhibition of angiotensin II/p38/SIRT1-induced podocyte apoptosis**

Gu, Junhui ; Yang, Ming ; Qi, Na ; Mei, Shuqin ; Chen, Jiejian ; Song, Shuwei ; Jing, Ying ; Chen, Meihan ; He, Liangliang ; Sun, Lijun ; Hu, Huimin ; Li, Lin ; Wüthrich, Rudolf P ; Wu, Ming ; Mei, Changlin

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**Original Paper**

# Olmesartan Prevents Microalbuminuria in db/db Diabetic Mice Through Inhibition of Angiotensin II/p38/SIRT1-Induced Podocyte Apoptosis

Junhui Gu<sup>a</sup> Ming Yang<sup>a</sup> Na Qi<sup>a</sup> Shuqin Mei<sup>a</sup> Jiejian Chen<sup>a</sup> Shuwei Song<sup>a</sup>  
Ying Jing<sup>a</sup> Meihan Chen<sup>a</sup> Liangliang He<sup>a</sup> Lijun Sun<sup>a</sup> Huimin Hu<sup>a</sup> Lin Li<sup>a</sup>  
Rudolf P. Wüthrich<sup>b</sup> Ming Wu<sup>a</sup> Changlin Mei<sup>a</sup>

<sup>a</sup>Kidney Institute, Department of Nephrology, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, China; <sup>b</sup>Division of Nephrology, University Hospital, Zurich, Switzerland

## Key Words

Diabetic nephropathy • Olmesartan • SIRT1 • Podocytes • Apoptosis

## Abstract

**Background/Aims:** Blockage of the renin-angiotensin II system (RAS) prevents or delays albuminuria in diabetic patients. The aim of this study was to investigate the inhibitory mechanism of the angiotensin receptor blocker olmesartan on albuminuria in a murine model of diabetic nephropathy. **Methods:** Male db/db diabetic mice were fed with placebo or 20 mg/kg olmesartan by daily gavage for 12 weeks. Conditionally immortalized mouse podocytes were treated with glucose, angiotensin II, olmesartan or p38 inhibitor s8307 in different experimental conditions after differentiation. **Results:** Olmesartan reduced albuminuria in db/db mice without change in body weight and glycemia. The increase of apoptotic cells and decrease of podocytes in the diabetic glomerulus were prevented by olmesartan. Moreover, olmesartan restored silent mating type information regulation 1 (SIRT1) expression in diabetic glomeruli. Furthermore, olmesartan treatment suppressed p38 phosphorylation but did not restore adenosine 5'-monophosphate-activated protein kinase (AMPK) phosphorylation in the diabetic glomerulus. *In vitro* study revealed that olmesartan prevented angiotensin II/p38/SIRT1 induced podocyte apoptosis, but it only slightly prevented high glucose/AMPK/SIRT1 induced podocyte apoptosis. In addition, the p38 inhibitor s8307 reversed SIRT1 expression and angiotensin II induced podocyte apoptosis. **Conclusions:** Olmesartan reduced albuminuria in diabetic nephropathy through inhibiting angiotensin II/p38/SIRT1 triggered podocyte apoptosis.

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J. Gu, M. Yang and N. Qi contributed equally to this paper and therefore share first authorship.

Prof. Changlin Mei  
and Dr. Ming Wu

Kidney Institute, Department of Nephrology, Shanghai Changzheng Hospital, No. 415  
Fengyang Road, Shanghai 200003 (PR China) Fax (0086)-21-63521416,  
E-Mail chlmei1954@126.com and mingwunl@126.com

## Introduction

Diabetic nephropathy is a leading cause of end stage renal failure, accounting for up to 40% of patients undergoing renal replacement therapy [1, 2]. Hypertension, proteinuria and progressive renal failure are most common clinical features of diabetic nephropathy [1, 2]. Animal and clinical studies demonstrated that blockage of the renin-angiotensin system (RAS) with angiotensin II receptor blockers (ARBs) prevented or delayed albuminuria in diabetic nephropathy independently of blood pressure control [3-5]. However the underlying mechanism of this beneficial effect remains to be elucidated.

Podocytes play a critical role in albuminuria development in diabetic nephropathy [6, 7]. Loss of podocytes correlated with increases in albuminuria in different diabetic animal models [7]. Since podocytes are terminally differentiated and non-renewable cells, treatments with renal protective agents did not rescue albumin leakage in diabetic animals with established albuminuria where podocytes have been lost [7].

The NAD<sup>+</sup>-dependent deacetylase Silent mating type information regulation 1 (SIRT1) is implicated in the pathogenesis of various diseases, including renal diseases [8, 9]. Thus, it has been shown that activation of Adenosine 5'-monophosphate-activated protein kinase (AMPK) by resveratrol decreased albuminuria in diabetic mice through SIRT1 [10]. In cardiovascular disease, p38 Mitogen-Activated Protein Kinase (MAPK) inhibition increased SIRT1 expression and thus prevented endothelial dysfunction [11].

SIRT1 is critical for podocyte viability, probably through modulating the activity of transcriptional factors such as p53 [6, 12]. Genetic deletion of podocyte SIRT1 reduced podocyte number and impaired their function, leading to increased albuminuria in diabetic mice through regulating acetylation status of transcription factors [6]. Angiotensin II can inhibit SIRT1 expression and induce apoptosis through activation of p38 in endothelial cells [11]. Therefore we hypothesized that angiotensin II increases albuminuria in diabetic nephropathy through p38/SIRT1 triggered podocyte apoptosis. Recent study shows that grape seed procyanidin B2 attenuates high glucose induced podocyte apoptosis through activating the AMPK/SIRT1 pathway [13]. It seems that the RAS and high glucose induce podocyte apoptosis through different pathways, and these two pathways may converge at the level of SIRT1.

Interestingly, a sub-analysis of the ROADMAP study showed that the ARB olmesartan inhibited albuminuria only in diabetic patients with higher blood pressure or with better glycemic control, suggesting that the RAS and high blood glucose induce albuminuria through different pathways [4]. We therefore hypothesized that olmesartan selectively inhibits angiotensin II induced podocyte apoptosis via the p38/SIRT1 pathway, but does not inhibit high glucose induced podocyte apoptosis. In the current study we aimed to test this hypothesis in db/db diabetic mice and in an *in vitro* culture model with olmesartan.

## Materials and Methods

### Animal Studies

10 to 12-week old male db/db diabetic mice with background strain C57BL/KsJ and their age-matched non-diabetic lean control mice (C57BL) were obtained from Shanghai SLAC laboratory Animal Co., Ltd. Mice were kept under local regulation and guidelines. The experimental protocol of the current study was approved by the Animal Care Committee at the Second Military Medical University, Shanghai.

10 non-diabetic control mice and 10 diabetic mice were fed with placebo (0.5% sodium CMC/saline solution), and 10 diabetic mice were fed with 20 mg/kg olmesartan (MB5704, Melone Pharmaceutical Co., Ltd, Dalian, China) by daily gavage for 12 weeks.

Mice were monitored for blood glucose, body weight and urine output every two weeks. After treatment, mice were euthanized and trunk blood was collected and was centrifuged to obtain plasma which was aliquoted and stored at -80°C. Kidney tissues were removed from mice. For protein extraction slices of

the kidney tissue were frozen in liquid nitrogen, and stored at -80°C. Other parts of the kidney tissue were fixed with 4% paraformaldehyde and embedded in paraffin for immunostaining.

#### *Blood glucose level test*

Fasting blood glucose (6-hour fast, blood taken from the tail vein) was measured using a glucose meter (ACCU-CHEK® Active, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Values were expressed in mmol/L.

#### *Urine collection and urinary albumin assay*

Mice were placed individually in metabolic cages for 24-hour urine collection with free access to food and water. Urine samples were centrifuged to remove debris and supernatants were aliquoted and stored at -80°C for later use. Quantitative urinary albumin was measured using a mouse ELISA kit (Sigma, USA).

#### *Immunohistochemistry and TUNEL Staining*

Immunohistochemistry staining for WT1 (Santa Cruz, SC-192), p-p38 (CST, 9211s), p-AMPK (CST, 2535s) and SIRT1 (Santa Cruz, SC-15404) was performed on 3-µm-thick tissue sections. In brief, the tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in an autoclave oven. After applying the primary antibody for 1 h, the sections were washed and then incubated with biotinylated secondary antibody (Vector) for 30 min. This was followed by application of the ABC reagent (Vector). Diaminobenzidine with metal enhancement was used as the detection reagent.

Apoptosis was identified within glomeruli by terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) using a commercially available kit (Roche, cat.no.11 684 817 910) according to the manufacturer's protocol.

The WT1, p-p38, p-AMPK, SIRT1 and TUNEL staining was quantified by counting the number of positively stained cells per glomerulus. At least 30 glomeruli per mouse were examined at 400x magnification.

#### *Podocyte Culture*

Conditionally immortalized mouse podocytes were obtained from Nanjing Medical University and cultured as previously described [14]. Briefly, cells were cultured with RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS, Gibco) at 33°C to propagate. To induce differentiation, podocytes were maintained without interferon-γ at 37°C for 14 days. Differentiated podocytes were treated with glucose, angiotensin II (MB1677, Melone Pharmaceutical Co., Ltd), olmesartan (MB5704) or p38 inhibitor s8307 (SB203580, Sigma) in different experimental conditions.

#### *Flow cytometry measurement of apoptosis*

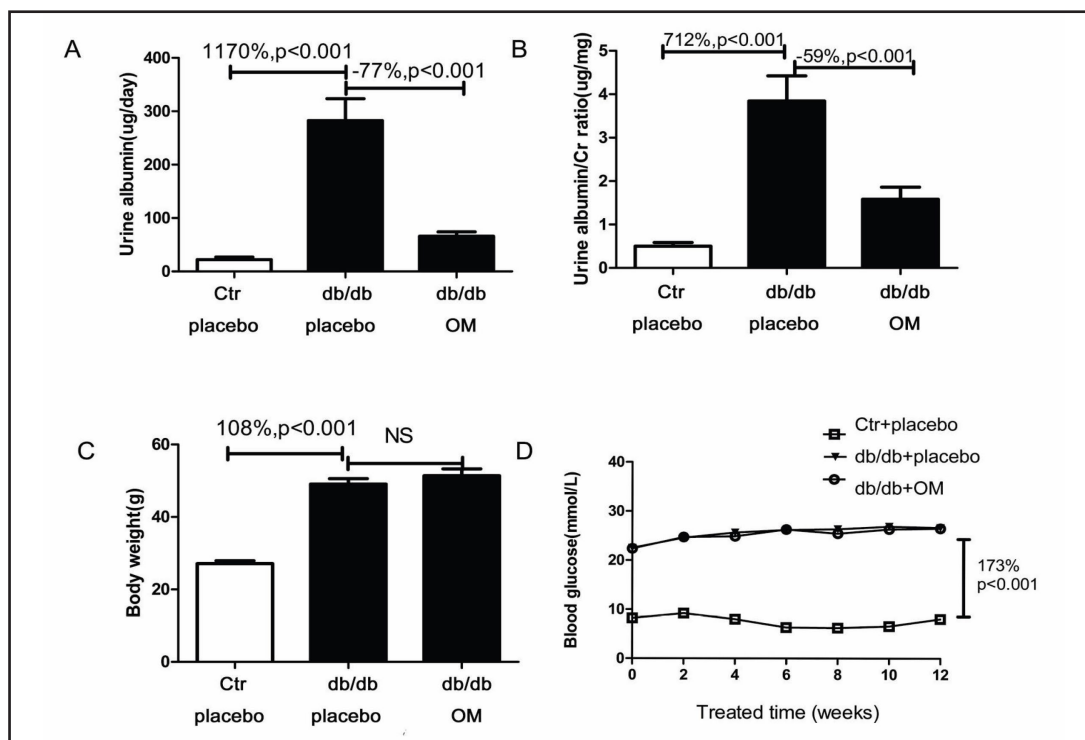
Podocytes were treated with glucose (5.5 mM or 30 mM), Ang-II (1 µM) or olmesartan (1 µM). After 24 hours of treatment, podocytes were harvested and stained with FITC-annexin V and PI (BD Biosciences) in binding buffer for 15 minutes in the dark. Stained cells were immediately subjected to flow cytometry analyses.

#### *Lactate dehydrogenase (LDH) assay*

Cell lines were treated with selected concentrations of glucose (5.5, 20 and 30 mM), Ang-II (1 and 10 µM) and olmesartan (1 and 10 µM). LDH enzyme release was measured using a CytoTox 96® non-radioactive cytotoxicity assay (Promega) according to the instructions provided by the manufacturer.

#### *Protein Extraction and Western Blot Analysis*

Snap frozen kidney tissue was homogenized in freshly made tissue protein extraction reagent (T-PER, Pierce Bioscience, Rockford, IL) containing 1 mM PMSF, 0.5 M EDTA, Halt Protease Inhibitor cocktail, and Halt Phosphatase Inhibitor. Homogenates were centrifuged twice at 4°C at 13,000 g for 15 min, and supernatants were stored at -80°C. To extract cell protein, cultured cells were washed with cold PBS and lysed in the same way. The lysate was centrifuged at 13,000g and 4°C for 15 min. The supernatant was collected and the concentration of total soluble protein was quantified using a BCA protein assay kit (Pierce, Rockford, IL). Lysates in SDS-sample buffer were boiled for 5 min at 95°C and equal protein amounts were resolved



**Fig. 1.** Efficacy of olmesartan in diabetic mice. A. B. C. Urine albumin, urine albumin/creatinine ratio and body weight were measured in control and db/db mice after 12 weeks treatment with olmesartan or placebo starting from week 10 to 12. D. Blood glucose was measured for a period of 12 weeks in control and db/db mice.

by SDS-PAGE gels before transferring to a PVDF membrane. After transfer, the PVDF membrane was sliced horizontally into pieces using prestained marker, and then followed by 1 h blocking at room temperature in blocking buffer (3% BSA in PBS with 0.05% Tween-20). Then, each separate blot was incubated for 16 h with first antibody in 3% BSA PBS-Tween. Each blot was washed and incubated for 1 h at room temperature with a secondary antibody and then visualized by enhanced chemiluminescence detection reagents. If two proteins were similar in size, two parallel blots were generated. The primary antibodies used for Western blot in this study were: SIRT1 (Santa Cruz, SC-15404), P-AMPK (CST, 2535s), AMPK (CST, 2532s), P-p38 (CST, 9211s), P38 (Santa Cruz, SC-7149), PARP (CST, 9542), AC-p53 (CST, 2590s), Bax (Affinity, AF0120), Bcl-2 (Affinity, AF9103), GAPDH (Santa Cruz, SC-365062). The band densities were measured using Image lab 4 software (Bio-Rad, USA).

#### Statistical analysis

Statistical analyses were performed by one-way ANOVA with the Newman-Keuls post hoc test using GraphPad Prism version 5.0 (GraphPad, San Diego, CA). All data are expressed as means  $\pm$  SD, and  $P < 0.05$  was considered as statistically significant.

## Results

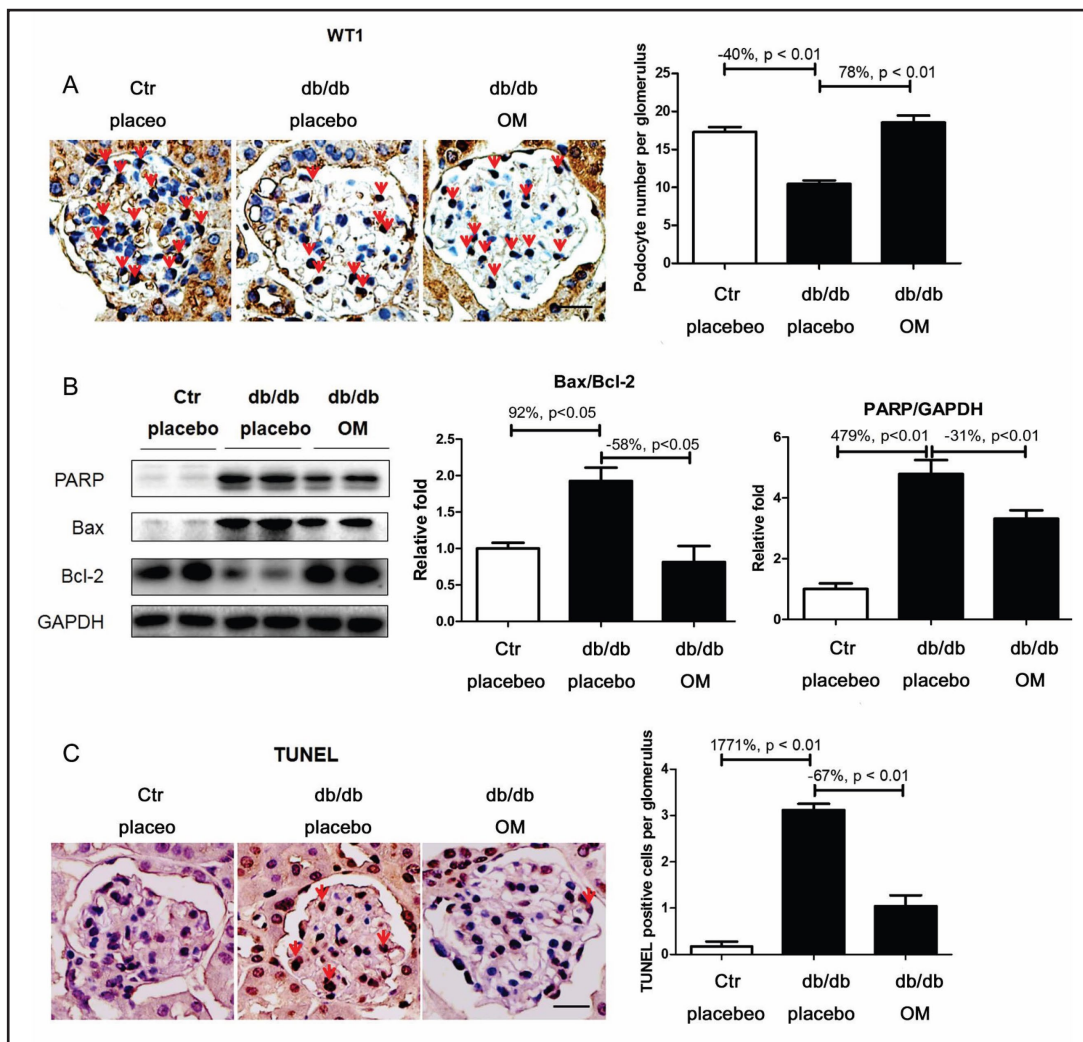
### *Olmesartan prevented microalbuminuria in db/db mice*

The efficacy of olmesartan was studied in db/db diabetic mice for a period of 12 weeks starting from week 10 to 12 of age. Figure 1A shows that db/db mice had 11.7 fold increased albuminuria in comparison to control mice at week 22 to 24 of age. Twelve weeks olmesartan administration significantly reduced albuminuria in db/db mice by 77% as compared with

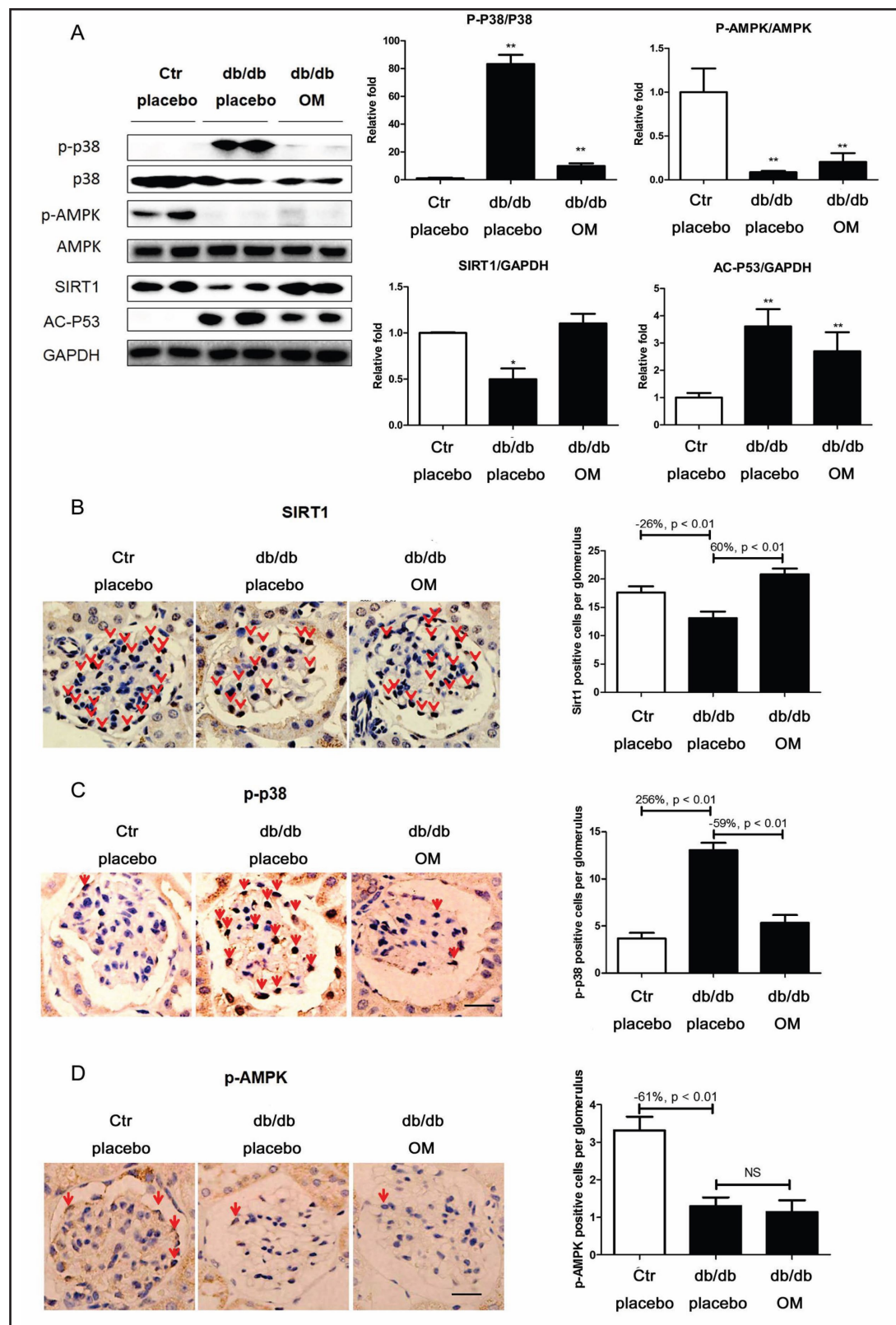


placebo treated db/db mice (Figure 1A). The albumin/creatinine ratio (ACR) was increased in db/db mice in comparison to control mice by 7.1 fold and olmesartan treatment significantly decreased ACR by 59% in db/db mice (Figure 1B).

In comparison to control mice, db/db mice had significantly increased body weight and olmesartan did not change the body weight of db/db mice (Figure 1C). In comparison to control mice, the blood glucose level was significantly elevated in db/db mice at week 10 to 12 of age when the experiment started and slowly increased till the end of the study, whereas olmesartan treatment did not change the blood level of glucose in db/db mice (Figure 1D).



**Fig. 2.** Effect of olmesartan on podocyte number and apoptosis in diabetic kidneys. A. Kidney sections from control and db/db mice were stained for WT-1 (Wilms tumour protein-1) to determine the podocyte number. Bar = 200  $\mu$ m. Arrowheads indicate cells which were positively stained. Quantification of glomerular number of WT-1 positive podocytes was shown in the lower panel. B. Western blot analysis of Bax, Bcl-2, and cleaved PARP protein expression in kidneys of control and db/db mice. The ratio of Bax/Bcl-2 was analysed by densitometry. C. Apoptosis assessed by TUNEL assay in glomerulus of control and db/db mice. Bar = 200  $\mu$ m. Arrowheads indicate cells which were positively stained. Quantification of glomerular number of TUNEL positive apoptotic cells was shown in the lower panel. Blots are representative of three independent experiments.



**Fig. 3.** Effect of olmesartan on protein expression in diabetic kidneys. A. Western blot analysis of p-p38, p38, p-AMPK, AMPK, SIRT1 and AC-p53 in kidneys of control and db/db mice were quantified by densitometry.



\*P<0.05 versus ctr+placebo, \*\*P<0.01 versus ctr+placebo. B. C. D. Immunohistochemistry analysis of SIRT1, p-p38 and p-AMPK in glomerulus of control and db/db mice. Bar = 200  $\mu$ m. Arrowheads indicate cells which were positively stained. Quantification of glomerular number of SIRT1, p-p38 and p-AMPK positive cells was shown in the lower panel of each picture. Blots are representative of three independent experiments.

#### *Olmesartan reversed podocyte number and glomerular apoptotic cell number in db/db mouse kidneys*

Wilms tumour protein-1 (WT-1) is a marker for podocytes. Figure 2A shows that the number of WT-1 positive cells was reduced in diabetic glomeruli by 40% in comparison to control and recovered by olmesartan treatment by 78%, suggesting that olmesartan prevented podocyte loss in diabetic kidneys.

The effect of olmesartan on apoptosis in kidneys was evaluated by Western blot and TUNEL staining. The cleaved PARP protein was increased in kidneys of db/db mice, which was attenuated by olmesartan treatment (Figure 2B). Moreover, the increase in Bax/Bcl-2 ratio in diabetic kidneys was significantly reduced by olmesartan treatment (Figure 2B). Furthermore, TUNEL staining showed that apoptotic cells were increased by 17.7 fold in the glomerulus of db/db mice as compared to control, and reduced by 67% by olmesartan treatment (Figure 2C). These data suggest that apoptosis was suppressed by olmesartan in diabetic kidneys.

#### *Olmesartan increased SIRT1 activity coupled with down-regulation of p-p38 in diabetic kidneys*

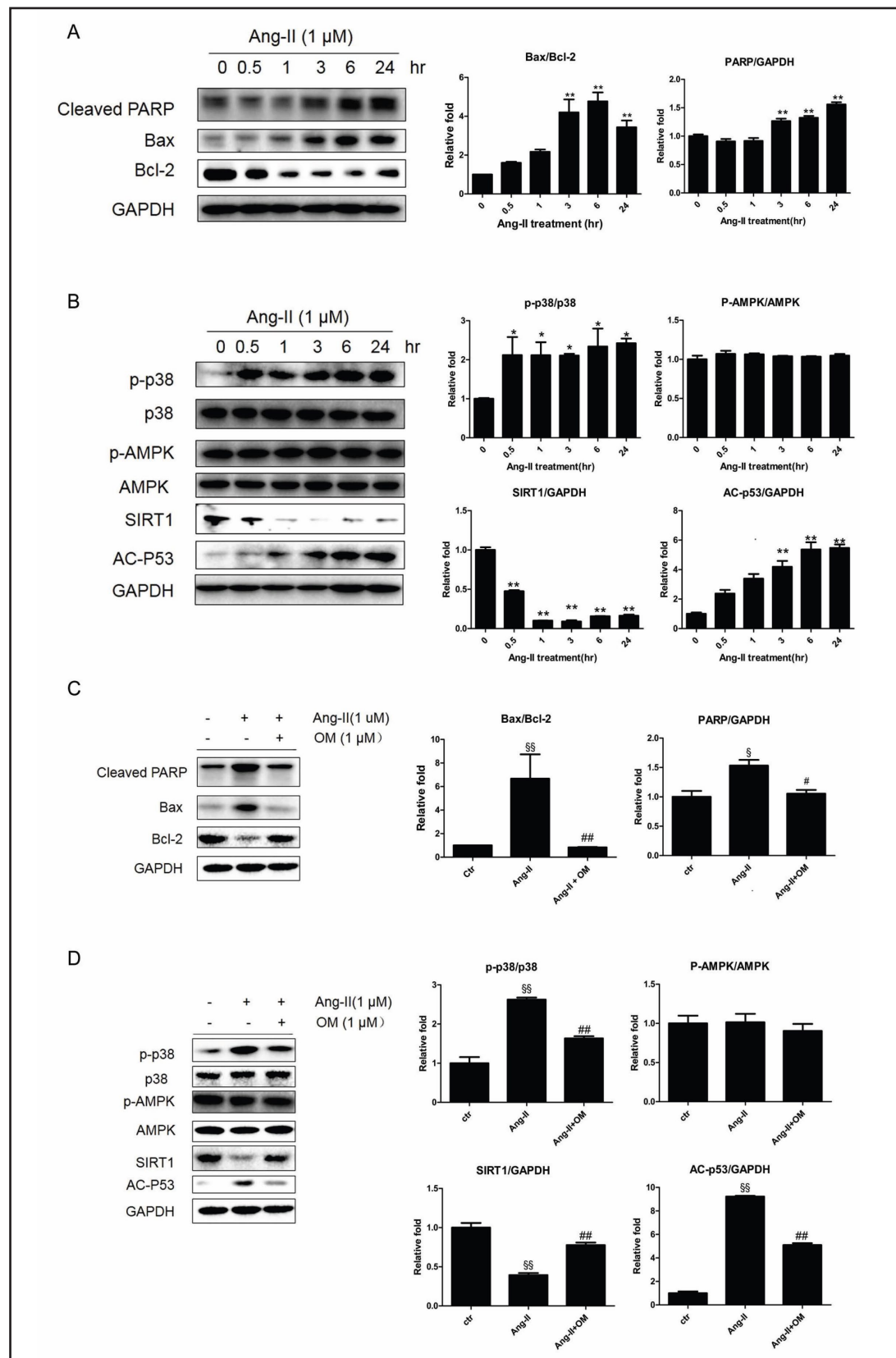
In comparison to control mice, SIRT1 was down-regulated in kidneys of db/db mice and its expression was restored by olmesartan treatment (Figure 3A). P53 is a downstream target of SIRT1 which was assessed by Western blot. Diabetic kidneys had increased acetylated p53 (AC-p53), while olmesartan treatment reduced p53 acetylation levels (Figure 3A). The activity of p38 MAPK and AMPK, two upstream regulators of SIRT1, were then evaluated. The amount of phosphorylated p38 was increased, whereas the AMPK phosphorylation levels were decreased in diabetic kidneys (Figure 3A). Olmesartan strongly reduced p-p38 but did not increase p-AMPK in diabetic kidneys (Figure 3A). Total p38 and AMPK protein were not changed in each group (Figure 3A).

The levels of SIRT1, p-p38 and p-AMPK in glomeruli was also analyzed by immunohistochemistry staining. Figure 3B shows that the number of SIRT1 positive cells was decreased in diabetic glomeruli by 26% in comparison to control mice, and restored by olmesartan treatment by 60%. In comparison to control mice, the number of p-p38 positive cells in diabetic glomeruli was increased by 2.6 fold, and was decreased by 59% by olmesartan treatment (Figure 3C). Figure 3D shows that the number of p-AMPK positive cells in diabetic kidneys was decreased by 61% in comparison to control mice, and was not changed after olmesartan treatment.

#### *Olmesartan inhibited angiotensin II induced podocyte apoptosis via p38/SIRT1 pathway*

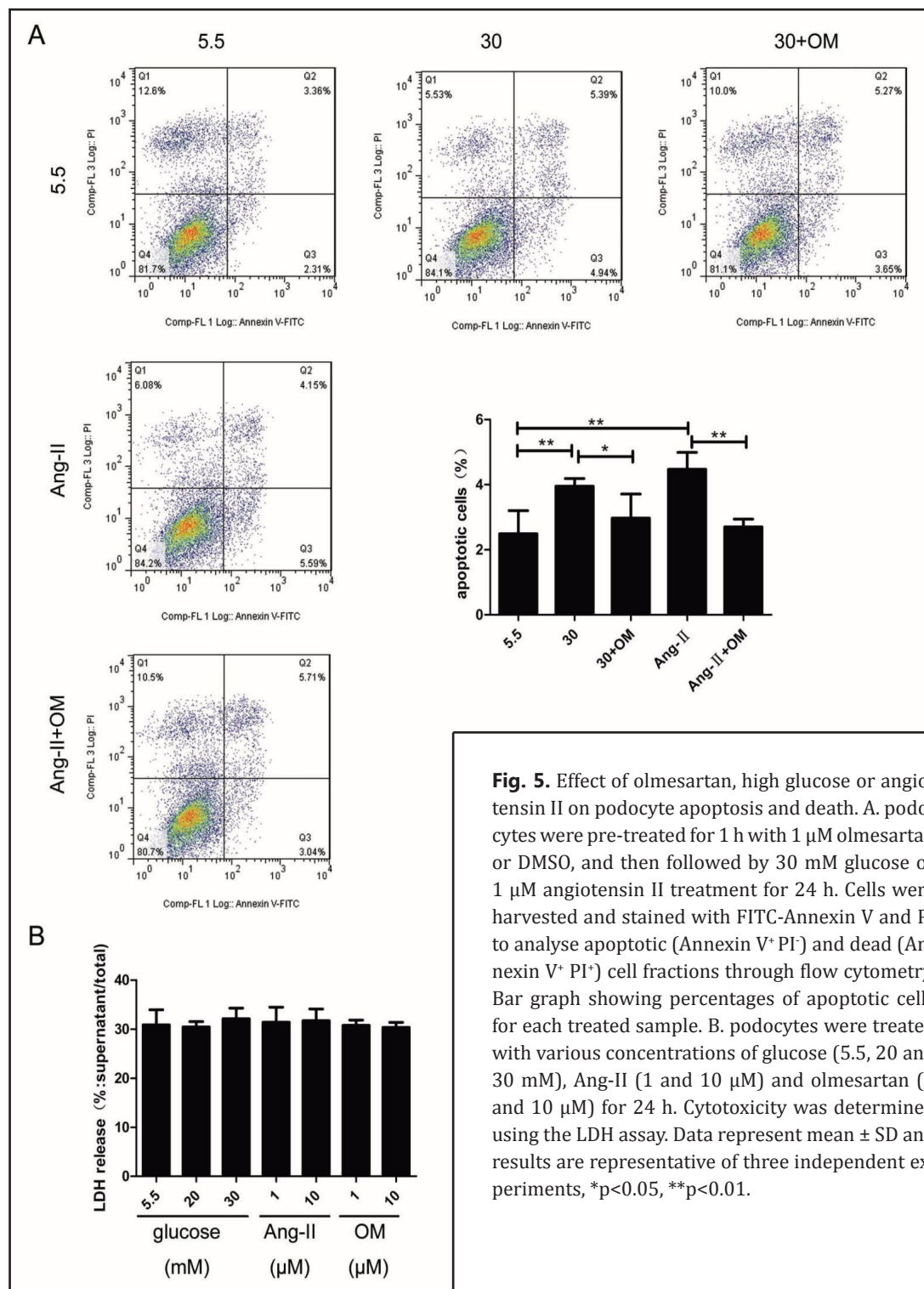
Figure 4A shows that the cleaved PARP protein and the Bax/Bcl2 ratio were increased in a time dependent manner in response to 1  $\mu$ M angiotensin II treatment of cultured podocytes, suggesting that apoptosis was induced in podocytes. Angiotensin II time dependently reduced SIRT1 expression and increased the levels of acetylated p53 (Figure 4B). Phosphorylation of p38 was increased by angiotensin II, whereas p-AMPK was not changed (Figure 4B). Total p38 and AMPK protein were not changed by 1  $\mu$ M angiotensin II treatment within 24 h.

Olmesartan reversed the increased ratio of Bax/Bcl2 and cleaved PARP protein expression in podocytes upon angiotensin II treatment (Figure 4C). Down-regulation of SIRT1 and up-regulation of AC-p53 triggered by angiotensin II was also reversed by olmesartan (Figure 4D). Increased p-p38 in angiotensin II treated podocytes was down-regulated by olmesartan



**Fig. 4.** Effect of olmesartan on angiotensin II induced podocyte apoptosis and protein expression. A. B. po-

podocytes were treated with 1  $\mu$ M angiotensin II (Ang-II) for different time. C. D. podocytes were pre-treated for 1 h with 1  $\mu$ M olmesartan or DMSO, and then treated with 0  $\mu$ M or 1  $\mu$ M Ang-II for 6h. Western blot analysis of p-p38, p38, p-AMPK, AMPK, SIRT1, AC-p53, Bax, Bcl-2, and cleaved PARP protein were quantified by densitometry. Blots are representative of three independent experiments. \* $P$ <0.05 versus 0 h, \*\* $P$ <0.01 versus 0 h, § $P$ <0.05 versus Ctr group, §§ $P$ <0.01 versus Ctr group, # $P$ <0.05 versus Ang-II group, ## $P$ <0.01 versus Ang-II group.



**Fig. 5.** Effect of olmesartan, high glucose or angiotensin II on podocyte apoptosis and death. A. podocytes were pre-treated for 1 h with 1  $\mu$ M olmesartan or DMSO, and then followed by 30 mM glucose or 1  $\mu$ M angiotensin II treatment for 24 h. Cells were harvested and stained with FITC-Annexin V and PI to analyse apoptotic (Annexin V<sup>+</sup> PI<sup>+</sup>) and dead (Annexin V<sup>+</sup> PI<sup>+</sup>) cell fractions through flow cytometry. Bar graph showing percentages of apoptotic cells for each treated sample. B. podocytes were treated with various concentrations of glucose (5.5, 20 and 30 mM), Ang-II (1 and 10  $\mu$ M) and olmesartan (1 and 10  $\mu$ M) for 24 h. Cytotoxicity was determined using the LDH assay. Data represent mean  $\pm$  SD and results are representative of three independent experiments, \* $p$ <0.05, \*\* $p$ <0.01.

(Figure 4D). Total p38 and AMPK expression and phosphorylation levels of AMPK were not affected by olmesartan in angiotensin II treated podocytes (Figure 4D).

The effect of angiotensin II and olmesartan on podocyte apoptosis were further confirmed by flow cytometry analysis. The number of apoptotic (annexin V+ PI-) podocytes were significantly increased by 1  $\mu$ M angiotensin II treatment, and 1  $\mu$ M olmesartan reduced the apoptosis by 40% in angiotensin II treated podocytes (Figure 5A). The toxic effect of angiotensin II and olmesartan on podocytes were assessed by lactate dehydrogenase (LDH) assay. Figure 5B shows that 31 % of LDH was released by control cells and angiotensin II (1 and 10  $\mu$ M) or olmesartan (1 and 10  $\mu$ M) had no effect on LDH release by podocytes.

*Olmesartan partly inhibited high glucose induced podocyte apoptosis which was triggered through AMPK/SIRT1 pathway*

Similarly, 30 mM glucose induced apoptosis in podocytes as indicated by time dependently increased expression of the cleaved PARP protein and increased Bax/Bcl-2 ratio (Figure 6A). SIRT1 was time dependently down-regulated and AC-p53 was up-regulated by 30 mM glucose treatment in podocytes (Figure 6B). P-p38 was not changed but p-AMPK was decreased by high glucose treatment (Figure 6B). Total p38 and AMPK were not changed throughout the experiment (Figure 6B).

Olmesartan partly ameliorated high glucose triggered podocyte apoptosis as shown by partly reduced expression of cleaved PARP and Bax/Bcl-2 ratio (Figure 6C). Impaired SIRT1 activity in high glucose treated podocytes was mildly recovered by olmesartan as shown by slightly increased SIRT1 expression and decreased AC-p53 (Figure 6D). Olmesartan did not inhibit p38 phosphorylation but slightly increased phosphorylation levels of AMPK in high glucose treated podocytes (Figure 6D).

Flow cytometry analysis confirmed that high glucose significantly increased apoptotic podocytes compared with normal glucose treated podocytes, and 1  $\mu$ M olmesartan treatment reduced the apoptosis in high glucose treated podocytes by 25% (Figure 5A). The toxic effect of high glucose on podocyte was excluded by showing that LDH release by podocytes was not significantly changed by 20 and 30 mM glucose (Figure 5B).

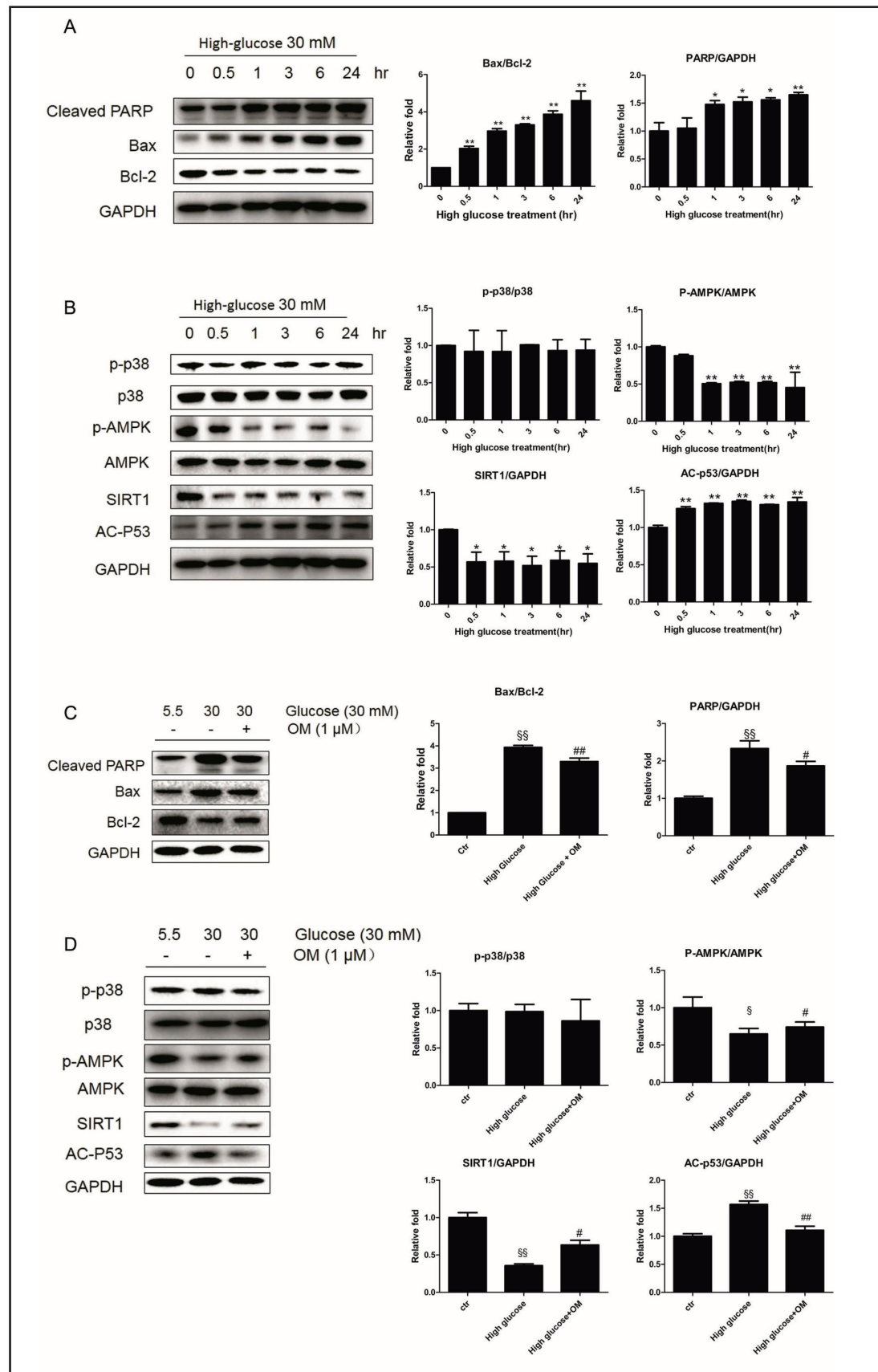
*Angiotensin II induced podocyte apoptosis through p38*

The ratio of Bax/Bcl-2 and the expression of cleaved PARP were decreased by the p38 inhibitor s8307 when used at a dosage of 2.5 to 25  $\mu$ M (Figure 7A). Figure 7B shows that s8307 decreased the levels of p38 phosphorylation and p53 acetylation and increased SIRT1 expression in podocytes in a dose-dependent manner.

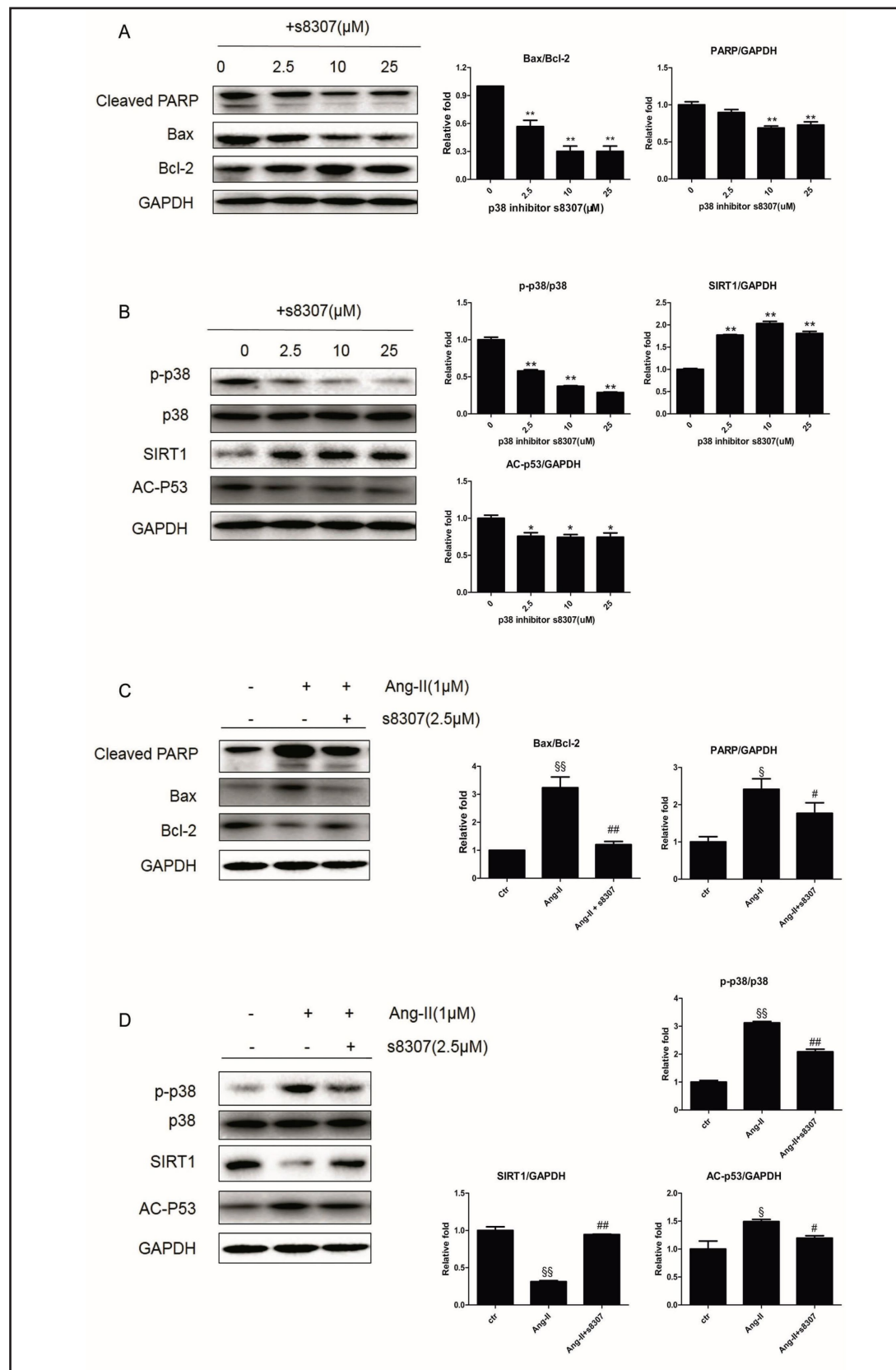
S8307 attenuated angiotensin II induced podocyte apoptosis as shown by reduced expression of cleaved PARP and reduced Bax/Bcl-2 ratio (Figure 7C). Figure 7D shows that 2.5  $\mu$ M s8307 reversed the enhanced phosphorylation levels of p38 in angiotensin II treated podocytes. Moreover, s8307 increased SIRT1 expression and diminished p53 acetylation in angiotensin II treated podocytes.

**Fig. 6.** Effect of olmesartan on high glucose induced podocyte apoptosis and protein expression. A. B. podocytes were treated with 30 mM glucose for different time. C. D. podocytes were pre-treated for 1 h with 1  $\mu$ M olmesartan or DMSO, and then followed by 5.5 mM (control) glucose and 30 mM (high) glucose treatment for 6 h. Western blot analysis of p-p38, p38, p-AMPK, AMPK, SIRT1, AC-p53, Bax, Bcl-2, and cleaved PARP protein were quantified by densitometry. Blots are representative of three independent experiments. \*P<0.05 versus 0 h, \*\*P<0.01 versus 0 h, §P<0.05 versus Ctr group, §§P<0.01 versus Ctr group, #P<0.05 versus high glucose group, ###P<0.01 versus high glucose group.



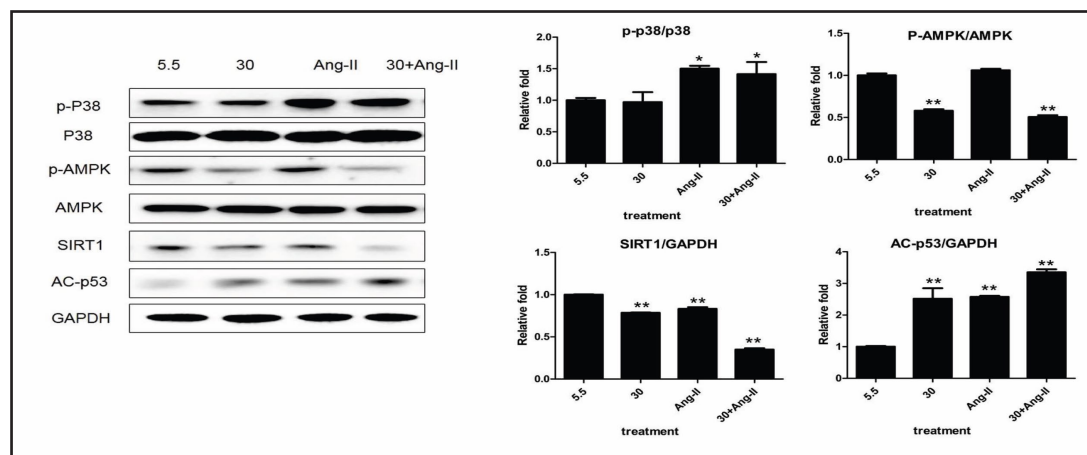






**Fig. 7.** Effect of p38 inhibitor s8307 on angiotensin II induced podocyte apoptosis and protein expression.

A. B. podocytes were treated with different concentration of s8307. C. D. podocytes were pre-treated for 1 h with 2.5  $\mu$ M s8307 or DMSO, then treated with 0  $\mu$ M or 1  $\mu$ M angiotensin II (Ang-II) for 6 h. Western blot analysis of p-p38, p38, p-AMPK, AMPK, SIRT1, AC-p53, Bax, Bcl-2, and cleaved PARP protein were quantified by densitometry. Blots are representative of three independent experiments. \* $P$ <0.05 versus DMSO Ctr group, \*\* $P$ <0.01 versus DMSO Ctr group,  $^{\S}$  $P$ <0.05 versus Ctr group,  $^{\S\S}$  $P$ <0.01 versus Ctr group,  $^{\#}$  $P$ <0.05 versus high glucose group,  $^{\#\#}$  $P$ <0.01 versus high glucose group.



**Fig. 8.** Combination of high glucose and angiotensin II on SIRT1 expression in podocytes. Podocytes were treated with glucose (5.5 mM, 30 mM), 1  $\mu$ M angiotensin II (Ang-II) and olmesartan (1  $\mu$ M). After 24 h of treatment, protein was collected. Western blot analysis of p-p38, p38, p-AMPK, AMPK, SIRT1, and AC-p53 were performed, and then quantified by densitometry. Blots are representative of three independent experiments. \* $P$ <0.05 versus 5.5 mM glucose treated control cells, \*\* $P$ <0.01 versus 5.5 mM glucose treated control cells.

### Combined effect of angiotensin II and high glucose on SIRT1 expression in podocytes

We next studied the combined effect of angiotensin II and high glucose on SIRT1 expression in cultured podocytes. Combined treatment of angiotensin II and 30 mM glucose did not further inhibit the phosphorylation of AMPK or increase the phosphorylation of p38 compared with treatment with high glucose or angiotensin II alone, whereas the combination of angiotensin II and 30 mM glucose further inhibited SIRT1 expression and increased acetylation of p53 (Figure 8). Thus angiotensin II and high glucose had an additive effect on the inhibition of SIRT1 expression in podocytes.

### Discussion

Albuminuria is an important risk factor for the progression of kidney and cardiovascular diseases [3, 4]. Therefore reducing albuminuria confers long-term protection against renal and cardiovascular diseases. It is generally accepted that targeting of the renin-angiotensin system (RAS) in diabetic diseases by angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) confers renal protection and reduces albuminuria independent of or in addition to blood pressure control [3-5]. In the ROADMAP study it was found that the ARB olmesartan prevented or delayed proteinuria in type2 diabetic patients [4]. However, the underlying mechanism of this anti-albuminuria effect is not completely understood. A growing body of evidence from animal studies demonstrated that loss of podocytes is correlated with increased albuminuria [6, 7, 15]. Podocytes are terminally differentiated cells and are unable to replace those lost. Many renal protective treatments

cannot reduce urinary albumin level in diabetic animals with established albuminuria, further highlighting the importance of podocytes in albuminuria development in diabetic nephropathy [7]. A large number of studies showed that the olmesartan and other ARBs prevented the loss of podocytes and reduced albuminuria in different model of renal diseases, suggesting that the RAS inhibition promotes podocyte survival [15-21]. In the present study we show that the reduction of albuminuria in db/db mice by olmesartan treatment associated with increased number of podocytes and decreased number of apoptotic glomerular cells in diabetic glomeruli. Furthermore our *in vitro* study shows that angiotensin II and high glucose induced podocyte apoptosis which could be blocked by olmesartan treatment. Taken together, our study further supports the protective effect of olmesartan on podocytes in diabetic nephropathy.

The NAD<sup>+</sup>-dependent deacetylase SIRT1 emerges as a new target and confers protective effects in several renal diseases, including diabetic nephropathy [6, 12]. The specific role of SIRT1 in podocytes in the context of diabetic nephropathy was elucidated recently [6]. Specific deletion of SIRT1 in podocytes increased podocyte loss and worsened albuminuria through enhancing the acetylation status and activation of downstream transcription factors [6]. Angiotensin II decreases SIRT1 expression in endothelial cells [11, 22]. Therefore we hypothesized that angiotensin II blockade by olmesartan prevents albuminuria in diabetic nephropathy through modulating SIRT1 mediated podocyte viability. Indeed, in our study we demonstrate that the dysregulated SIRT1 expression and acetylation of its downstream targets in diabetic kidneys were reversed by olmesartan treatment. Moreover, the increase of SIRT1 positive glomerular cells correlated with ameliorated glomerular apoptosis and albuminuria in db/db mice after olmesartan treatment. Our *in vitro* study further showed that olmesartan prevented podocyte apoptosis which was associated with restored SIRT1 expression. Besides its effect on apoptosis, SIRT1 regulates a large array of cellular processes through deacetylation of transcriptional factors such as NF- $\kappa$ B dependent inflammatory responses and PGC-1 $\alpha$  mediated oxidation and mitochondrial biogenesis [23-25]. Since acetylation of NF- $\kappa$ B (p65) and PGC-1 $\alpha$  are increased in diabetic kidney, it would be interesting to investigate whether olmesartan reduces albuminuria in diabetic nephropathy through ameliorating SIRT1 mediated inflammation, oxidative stress and mitochondrial dysfunction [6, 10, 13].

High glucose and the advanced glycolysis end products (AGEs) can inhibit SIRT1 expression in renal cells [6, 26]. The blood glucose level was unchanged during our study, and therefore it is unlikely that olmesartan treatment increased SIRT1 expression via glycemic control. One limitation of our study was that blood pressure was not measured during the treatment. High blood pressure is a risk factor for diabetic nephropathy [27, 28]. In previous reports, olmesartan inhibited albuminuria in diabetic animals, but this effect was not tightly correlated with blood pressure control [16, 17, 20, 29]. Of course, we cannot exclude that the reduced blood pressure by olmesartan treatment contributed to the decreased albuminuria in db/db mice, however the focus of our study was to investigate the effect of olmesartan on angiotensin II/SIRT1 pathway triggered podocyte apoptosis.

Subgroup analysis of the ROADMAP study showed that olmesartan displayed significant anti-albuminuria effect only in diabetic patients with baseline blood pressure over 135 mm Hg or with better glycemic control, suggesting that high glucose and RAS induced albuminuria in separate pathways and blockage of RAS was not sufficient to manage hyperglycemia induced albuminuria [3, 4]. Furthermore, animal studies showed that only maximal blockage of RAS can reduce albuminuria in normotensive diabetic animals, implying that the sensitivity of RAS inhibition is dependent on the etiology of podocyte injury [4, 7]. It seems that angiotensin II down-regulates SIRT1 through a different pathway than high glucose or AGEs [11, 26]. Angiotensin II could inhibit SIRT1 expression through p38 MAPK, which is highly expressed in podocytes in diabetic kidneys and increased p38 phosphorylation was correlated with podocyte loss and increased albuminuria [11, 30]. Studies showed that inhibition of p38 increased SIRT1 expression because p38 directly interacts with SIRT1 and

promotes its degradation [11, 31]. In contrast, high glucose is less potent to enhance p38 activity in renal cells [32, 33]. AMPK is an energy sensor and it is quite sensitive to high glucose or AGEs stimulation [26, 34, 35]. High glucose reduces AMPK  $\alpha$ -subunit threonine (Thr) 172 phosphorylation, which determines the enzymatic activity of AMPK [36, 37]. Down-regulation of AMPK inhibited SIRT1 expression and associated with increased albuminuria, while activation of the AMPK/SIRT1 pathway inhibited high glucose induced podocyte apoptosis [13, 26, 38]. Two studies showed that AMPK may be downstream of angiotensin II [39, 40]. However, only minor change of phosphorylated AMPK was observed after a non-specific AT1 blocker treatment or after angiotensin II treatment in these two studies. Thus we hypothesize that angiotensin II blockade rescues angiotensin II/p38/SIRT1 induced podocyte apoptosis but not high glucose/AMPK/SIRT1 induced podocyte apoptosis. In the present work, we demonstrate that restoration of SIRT1 expression by olmesartan correlated with down-regulation of p38, and that there was no change in blood glucose and AMPK activity in db/db mice. Our *in vitro* study showed that angiotensin II induced podocyte apoptosis required p38 and was correlated with decreased SIRT1 expression. Moreover, olmesartan prevented podocyte apoptosis which was correlated with decreased p-p38 and increased SIRT1 expression. Furthermore, high glucose induced podocyte apoptosis was correlated with decreased AMPK phosphorylation levels and reduced SIRT1 expression, while high glucose had no effect on p38 activity. Finally, olmesartan only slightly restored AMPK activity and SIRT expression, and thus partly prevented high glucose induced podocyte apoptosis.

Interestingly, combination of angiotensin II and high glucose further inhibited SIRT1 expression in podocytes, probably through two different upstream pathways. Thus, targeting both p38 and AMPK pathway in diabetic nephropathy may provide additive protection against podocyte injuries, and this should be tested in the future study.

## Conclusion

Taken together, our study revealed a novel mechanism of olmesartan's effect on albuminuria in diabetic nephropathy, namely angiotensin II/p38/SIRT1 triggered podocyte apoptosis. Our study suggests that prevention of albuminuria in diabetic nephropathy could be specifically tailored to the etiology of podocyte apoptosis.

## Disclosure Statement

No conflict of interest was declared.

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